Chapter 1
Introduction: circadian rhythm and photopharmacology

In order to cope with daily temperature and light changes caused by rotation of the Earth around its axis, living organisms developed an adaptive endogenous regulation system called the circadian clock. Rhythmic 24 h changes in metabolism and cellular content happen throughout the whole mammalian body, and they are uniformly regulated in every cell. Keeping perfectly synchronized rhythms in mammals is crucial for health and homeostasis. Recently, the disruption of the circadian clock has been connected to a wide variety of diseases and disorders. However, a pharmacological approach to selectively 'fix' a piece of broken cogwheel machinery is challenged by the complex and uniform regulation of circadian oscillations throughout the mammalian body. Therefore, photopharmacology, an emerging field that utilizes light as the unmatched external stimulus to control the activity of small molecules, may offer a solution for a better understanding of circadian regulation as well as for gaining a spatio-temporal control over circadian rhythmicity.

1.1 Circadian rhythm

1.1.1 General introduction

In order to survive, living organisms had to develop a coping mechanism for large daily environmental changes (*e.g.,* light and temperature) caused by rotation of the Earth around its axis. Therefore, almost all living organisms ranging from bacteria to humans have an intrinsic time-keeping system that entrains daily rhythms of biological processes with the Earthly cycles.[1] The temporal synchronization of internal processes among themselves and with external changes is governed by circadian clocks.[2,3] Circadian clocks are robust endogenous biological oscillators with a period of approximately (in Latin - 'circa') one day ('dies').[4,5] The first demonstration of the internal clock existence was conducted by the French astronomer Jean Jacques d'Ortous de Mairan in 1729.[6] De Mairan placed a plant *Mimosa pudica* in the dark box and followed its behavior under constant light conditions. Despite the absence of light, the plant retained 24 h cycles of opening and closing leaves (Figure 1). This experiment revealed one of the intrinsic properties of the circadian clock – persistence under constant conditions driven by autonomous regulation.

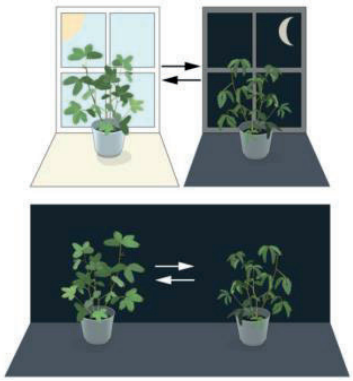


Figure 1. (A) De Mairan`s experiment on the *Mimosa pudica* demonstrating that it retained internal clock of the plant in constant darkness. Adapted from Copyright © 2017 The 2017 Nobel Prize in Physiology or Medicine.

Although the circadian clocks are endogenous, they are attuned (entrained) to the daily light-dark (LD) cycles. When the organism is not exposed to light, there is no entrainment to the astronomical time leading to 'free-running' circadian rhythms.[7] The period of 'free-running' rhythms in many living organisms differs from 24 h. For instance, in 'Andechs bunker experiment', when a group of people was isolated from an environment without perception of external time cues and free to choose their sleep/awake schedules, their internal circadian period started to run as 25.2 h long.[8,9] More recent studies of Czeisler *et al* showed with high precision that the intrinsic period of the human circadian clock averages 24.2 h.[10] The 'free-running' period of circadian rhythms in other organisms also deviates, being longer or shorter than 24 h.[11] Moreover, in humans, a modern life-style can impose time constraints that are mismatching with internal circadian physiology. For example, as a result of transmeridian traveling, misalignment of the internal and external

time causes jetlag.[12] However, exposure to the daily photic inputs resets (entrains) biological time, adjusting sleep/wake cycles to the new time-zone. Light, as well as non-photic external signals (such as food intake, temperature, physical activity, social interaction, medication, etc.) that synchronize the human biological clock with the Earth`s 24 h rotation, are called *zeitgeber*s ('time givers' or 'synchronizers').[13,14]

1.1.2 Hierarchical organization of mammalian circadian clocks

In mammals, rhythms of clock genes and protein expression have been observed in almost all cells throughout the body.[15–18] However, the organization of the clocks is highly hierarchical, being consisted of the 'master' clock and peripheral clocks (Figure 2).[19] As a main *zeitgeber*, light input is detected in the eyes by a recently discovered photoreceptor cell type called intrinsically photoreceptive retinal ganglion cell (ipRGC), which respond to blue light.[20] The photic input from ipRGCs is further phototransduced to the 'master' clock in the brain providing information about the time of the day – the intensity of blue light is high during the day and low during the night.[21] The 'master' clock or the circadian pacemaker is located in the suprachiasmatic nuclei (SCN), a 20 000-neurons-large bilateral region situated in the hypothalamus, just over (supra) the optic chiasm – the part of the brain where the nerves of the eyes cross.[22,23] Carrying the information of the astronomical time, SCN synchronizes the rhythms of peripheral clocks throughout the body.[24,25] Peripheral clocks are endogenous cellular rhythms situated in organs and tissues different from SCN.[15,26]

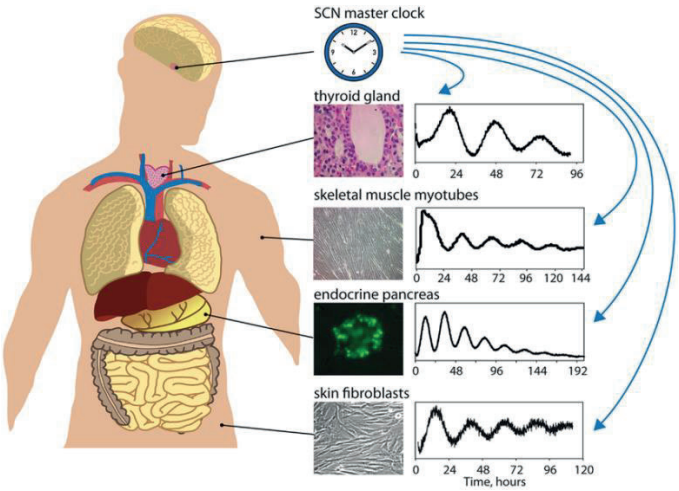


Figure 2. Hierarchical organization of mammalian clocks. SCN as a master clock synchronizes all other, peripheral clocks. Adapted with permission from Ref. [27] Copyright © 2017 American Association for the Advancement of Science.

The initial belief was that circadian oscillations of peripheral clocks are fully dependent on the SCN, but the discovery of the 'clock genes' enabled the use of gene reporter methods and showed that isolated peripheral organs and tissues can form stable oscillations on their own.[28] However, despite the identical molecular regulation of the circadian rhythmicity in

the brain and periphery, *in vitro* oscillations of peripheral clocks rapidly damp in the period of 3–6 days, while SCN tissues exhibited robust oscillations during a period of weeks.[29,30] This indicates that synchronization of peripheral clocks by SCN is required in order to sustain their oscillations. If there is no synchronization, cells in peripheral tissues become out of the phase, leading to a destructive interference and decrease in amplitude read-out.[16]

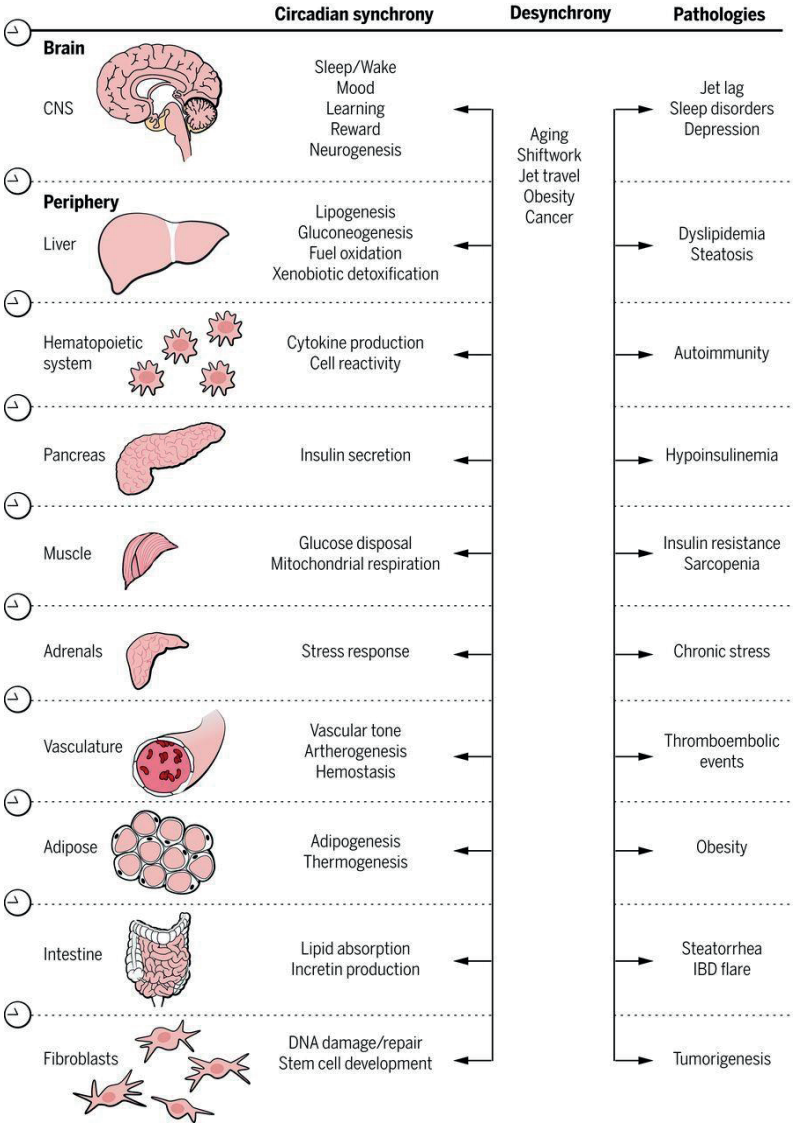


Figure 3. Diseases and disorder caused by disruption of the organ-specific circadian clock. Adapted with permission from Ref. [3] Copyright © 2016 American Association for the Advancement of Science.

Next to the 'master' clock synchronization, the peripheral clocks are additionally entrained by non-photic *zeitgebers*, mainly by food, social interactions or physical activity.[25,24,31] For instance, in the experiment with transgenic rats, when access to food was experimentally restricted only during the daytime, even though being nocturnal animals, they became active during the day.[30,32] Only after 2 d of restricted feeding, the circadian phase of the liver was entrained by a shift of 10 h, while food non-related tissue of lungs showed a shift of 4 h. On the other hand, rhythmicity in the SCN stayed unchanged and in synchrony with LD cycles. This suggests that non-photic *zeitgeber* such as food can uncouple peripheral oscillators from the central pacemaker, indicating a certain degree of peripheral clock autonomy and susceptibility to change. These findings highlighted the difficulty to maintain circadian order across organs and tissues, especially having in mind a modern life-style.

1.1.3 Therapeutic implications of disrupted circadian rhythms

A whole array of genes is rhythmically expressed in almost all the mammalian cells in order to retain homeostasis of the organism. The master clock is more responsible for general functions such as tuning mood and behaviour, while the peripheral clocks are the ones responsible for more specific functions such as hormone secretion, metabolism, heart rate, body temperature, etc.[33–35] Therefore, constant desynchrony from the environmental cues or genetic mutations of clock genes can lead to a disrupted steady-state of circadian homeostasis and cause organ-specific diseases or disruption in physiology of the organism (Figure 3).[36] One of the main causes of circadian related diseases and disorders lies in a modern life-style. Light-polluted environment, night-shifts, modern diet, obesity and transmeridian travel lead to desynchronization of biological time with external day-night cycles causing disruption of circadian clocks, mainly peripheral ones.

Circadian oscillations can be parameterized by period, amplitude, and phase, and each of these parameters can get disrupted (Figure 4A-D). Phase disruption shifts the whole oscillation pattern to early or late stages, causing, for instance, earlier or later sleeping times (Figure 4B). The disruption in amplitude lowers the magnitude of the response, for example leading to less intense secretion of hormones (Figure 4C). Disruption of the period consists of longer or shorter oscillation times, producing a desynchronization of the internal circadian time with the environmental light cycle (Figure 4D).[13,37] When isolated, the robustness of the clock is weak (particularly in peripheral tissues) and its amplitude quickly damps over time due to desynchronization among the cells (Figure 4E).

During the last two decades, numerous implications and connections of circadian clock disruptions with diseases and disorders development have been made. For instance, early discoveries showed that misalingment of feeding time with endogenous clock time caused obesity in mice.[38,39] On the other hand, alignement of food intake and activity protected rodents from a fatty liver[40] and improved metabolic health in humans.[41] Night-shift workers whose internal circadian rhythms are mismatched with LD cycles are prone to obesity,[42] metabolic diseases[43] and breast cancer.[44] Exposure to blue light at night or staying up late can induce similar effects in humans.[42,45] This effect is better known as social jetlag. Next to shiftwork conditions, clock synchronization decreases with aging in mammals.[46]

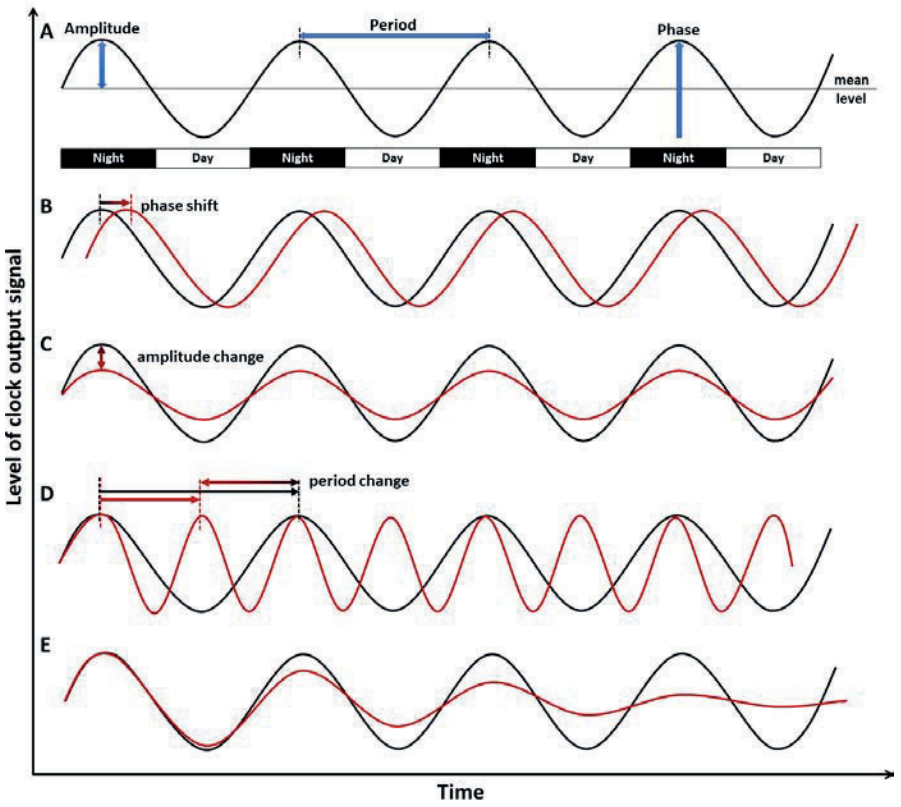


Figure 4. Properties of circadian oscillations. (A) Three circadian parameters: phase, amplitude and period. Phase is defined as the time between a reference point and a fixed event of the cycle, amplitude represents the magnitude of the read-out (*e.g.*, hormone secretion), and period describes the time between to reference points within one oscillation (*e.g.*, two amplitude maxima); (B) the phase shift where the red curve represents delayed phase; (C) the amplitude change caused by decreased read-out level; (D) the period change. Rhythms in red exhibit a period shortening; (E) a comparison between robust synchronized *in vivo* or SCN oscillations (black) and isolated oscillations of peripheral clocks (red). Under *in vitro* experiment conditions robustness is weak and dumps during the period of a couple of days.

Moreover, recent experimental and genetic studies revealed cell-specific pathways through which circadian disruption affects diseases and metabolic pathways.[3] Insulin secreation by pancreatic β-cells is a crucial physiological mechanism that narrowly maintains levels of glucose in healthy people. This process is enabled by β-cells` circadian clock which helps to anticipate the body`s varying need for insulin during the day.[47,48] Ablation of the pancreatic clock in mice causes severe glucose intolerance and diabetes mellitus, demonstrating the importance of the β-cells clock in coordinating insulin secretion with the sleep-wake cycle.[47,49] Peripheral clocks are also responsible for the rhythmic regulation of many liver functions, such as energy metabolism and enzyme expression.[50] A dysfunction of the liver

peripheral clock accelerates the development of liver diseases, such as fatty liver,[51] hepatitis,[52] cirrhosis[53,54] and liver cancer.[55,56] Chrononutrition emerged as a powerful method for keeping a liver homeostasis and can be more powerful than SCN-mediated regulation of the peripheral clocks.[50,57,58] Additionally, skin-related processes are circadian such as skin aging, cell repair, cancer development and drug delivery to the skin.[36]

In Alzheimer's disease, the phase of the circadian rhythm has been found to be dysregulated between various regions of the brain.[59] Furthermore, oscillations in the pineal gland, responsible for the secretion of melatonin, were disrupted, explaining the often disturbed sleep-wake pattern in patients with Alzheimer's disease.[60] Restoring the circadian rhythm by means of light therapy and melatonin supplementation displayed beneficial effects on memory and improved the cognitive state of patients with Alzheimer's.[60]

The recent discovery of Rijo-Ferreira *et al.* showed that sleeping sickness, caused by a unicellular *Trypanosoma brucei*, is also a circadian disorder.[61] Upon infection with *T. brucei*, this parasite triggers circadian period shortening and phase advance at the behavioral level as well as at the tissue and cell level. The authors proposed that the parasite causes an advance circadian rhythm disorder which was previously associated with mutations in clock genes. Additionally, it was found that the circadian clock of immune cells (primarily neutrophils and macrophages as the parasite host cells) influences the magnitude of infection by the parasite *Leishmania*.[62]

With all these and many other circadian related diseases in mind, it becomes clear that understanding a connection between circadian regulation and disease development is one of the crucial tasks to successfully utilize chronotherapy.

1.1.4 Mechanism of the circadian clock regulation in mammalians

As mentioned previously, circadian rhythms are autonomous biological oscillations present in almost every mammalian cell and their molecular regulation is uniform throughout the whole body.[16,63] On the cellular level, circadian rhythm is controlled by a negative feedback loop, so called 'core-clock loop'.[29,64] This negative feedback loop involves two main transcriptional activators: Circadian Locomotor Output Cycle Kaput (CLOCK) and Brain-Muscle-Arnt-Like protein 1 (BMAL1), as well as repressors period (PER) and cryptochrome (CRY). *Clock* mRNA and protein are constitutively produced in the SCN,[65] while *Bmal1* mRNA expression peaks in the middle of the circadian night. Rhythmical expression of *Bmal1* is controlled by *Rev-erbα*, a gene of another sub-loop,[5,66,67] which will not be taken into consideration due to the scope of this thesis. A heterodimeric transcriptional activator complex CLOCK-BMAL1 is formed in the nucleus and by acting on the key circadian E-box promotor element, it promotes transcription of the 'clock-controlled genes' (Ccg) – an assembly of genes that are responsible for various rhythmic biological processes.[63] This assembly also contains genes of their own repressors PER and CRY. *Per* and *Cry* mRNAs peak in the SCN in mid-to-late circadian day, regardless of whether an animal is nocturnal or diurnal, while PER and CRY peak about 4 h later.[68] Approximately 12 h after their transcriptional activation, PER and CRY form a large complex after accumulation in the cytoplasm, followed by translocation into the nucleus inhibiting CLOCK-BMAL1 transcription factors, repressing their own gene expression.[69,70]

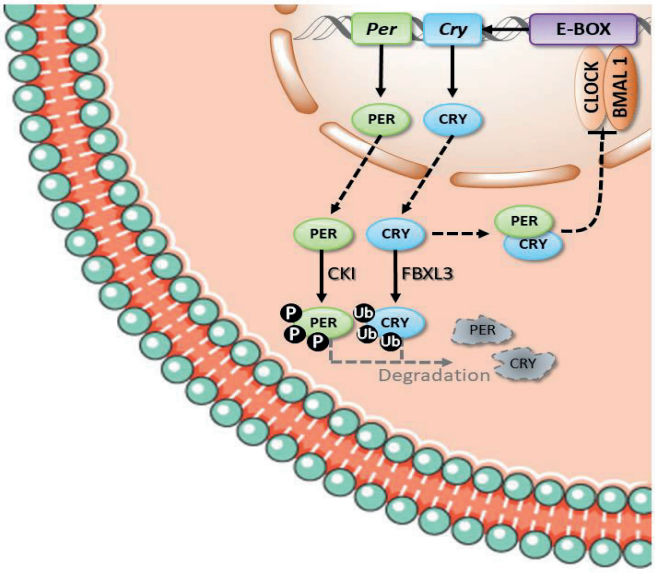


Figure 5. The circadian 'core-clock' negative feedback loop regulation.

One circadian oscillation takes about 24 h, and the turnover of the PER and CRY proteins is tightly regulated by post-translational modifications.[71] A family of casein kinase I (CKI, isoforms α, δ and ε) facilitate phosphorylation of PER while F-box and leucine-rich repeat protein 3 (FBXL3) performs the ubiquitination of CRY proteins. These processes promote degradation of PER and CRY by proteasomal pathway and therefore permitting a feedback loop to reactivate.[72] Recent studies have shown that approximately 80% of protein-encoding genes display circadian oscillation on the mRNA level.[73]

Disrupted circadian oscillations caused by mutations of core-clock or related genes have been associated to the development of numerous diseases and disorders.[5,74] For instance, hypophosphorylation of PER2 causes familial advance sleep phase syndrome (FASPS) characterized by 4 h advanced (shorter) sleep, temperature and melatonin rhythms.[74] Hypophosphorylation has been found to occur in hamsters as CK1ε mutation[75] or in humans with serine to glycine mutation within the CK1ε binding region of hPER2.[74] Similarly, in transgenic mice, mutation in a phosphorylation site within CK1δ also results in FASPS.[76] Furthermore, isoleucine to threonine mutation-caused substitution in FBXL3 leads to loss of its function and prevents recognition of CRY.[72] The loss of CRY recognition by FBXL3 induces CRY stabilization and consequently elongates the circadian period. Shown by recent studies, mutation of *Cry* gene is associated with delayed sleep phase disorder (DSPD),[77] diabetes[78,79] and cancer development.[80–83] Research by Young *et al* revealed that deletion of exon 11 in CRY1 DSPD allele leads to an increased CRY1 localization in the nucleus. This finding, together with previously demonstrated correlation of period lengthening upon enhanced CRY1 and BMAL1 interaction,[84] explained a late chronotype found in humans with DSPD.

1.1.5 Chronotherapy: introducing the circadian clock to pharmacology

Understanding of the molecular circadian clock regulation, as well as deeper insight into the connection between its disruption and disease or disorder development, paves the way for development of chronotherapy. Next to targeting essential components of the circadian feedback loop with drugs, new therapeutic frontiers are placing the circadian clock broadly in the central role in order to treat or prevent several chronic diseases.[85] Therefore, the emerging field of chronotherapy involves three major strategies:

1. optimizing the circadian lifestyle ('training the clock')
2. timing of drug administration ('clocking the drugs'), and
3. pharmacological targeting of circadian clock components ('drugging the clock').[85]

1.1.1.1 Training the clock

As previously mentioned, unnatural exposure to some of the *zeitgeber*s can lead to a misalignment of our internal time with the solar time causing circadian disorders.[43,44] However, restoring circadian rhythmicity by keeping a natural feeding-fasting, sleep-wake, or light-dark cycles can prevent development of chronic diseases.[85]

Light-pollution and constant presence of blue light-reach sources are considered as the major inducers of circadian disruption. When exposed to light at night, the sleep-promoting hormone melatonin is suppressed and delays sleep onset.[85–87] Therefore, reducing an evening exposure to light and increasing exposure to daylight helps keeping sleep-wake cycles entrained within 24 h rhythms.

As a major *zeitgeber* for peripheral rhythms, the timing of food intake can significantly modulate those rhythms. It is shown that even in absence of a functional circadian oscillator, time–restricted feeding can synchronize nutrition-sensing and utilization pathways (cAMP-response element binding protein, rapamycin kinase, Forkhead box O, etc.) in the liver.[40,88]

Keeping natural sleep-wake cycles is crucial in preventing pancreatic disorders. Melatonin is secreted from the pineal glands during the night-time and consequently suppresses insulin release. Thus, disturbed sleep-wake cycles may influence pancreatic function.[89–91]

1.1.1.2 Clock the drugs

More than 80% of targets of FDA-approved drugs obey circadian rhythmicity.[73,92] This means that the success of drug therapies highly depends on the time of administration. Due to general circadian rhythmicity of metabolism, pharmacokinetics and pharmacodynamics of drugs may also be improved by timing drug delivery to align it with the innate circadian rhythm (Figure 6).[92–95]

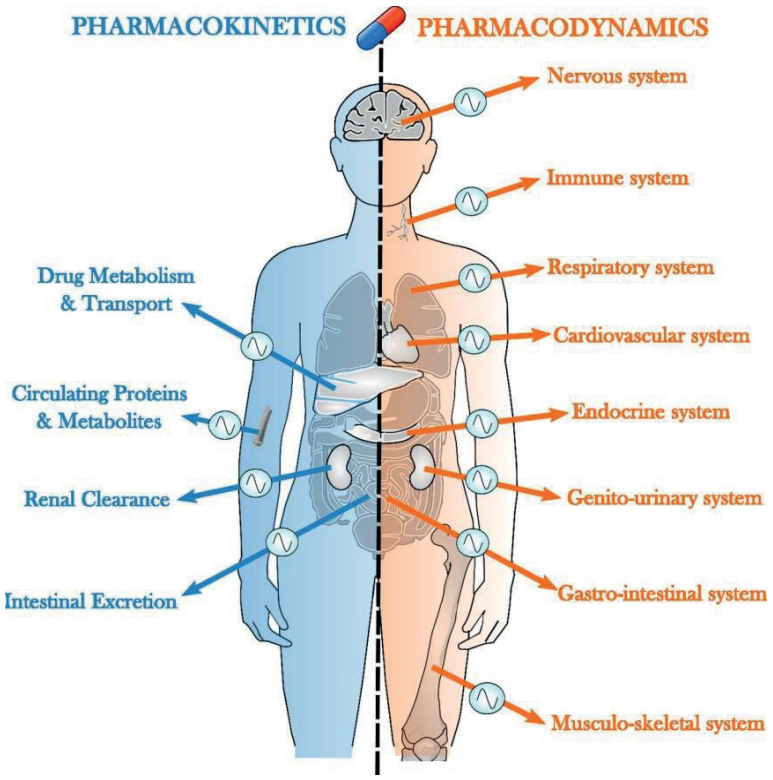


Figure 6. Pharmacokinetics and pharmacodynamics controlled by circadian rhythmicity. Processes like intestinal absorption, intracellular uptake and efflux, and renal and intestinal excretion are rhythmical. Even plasma proteins and blood metabolites that are binding to drugs vary during 24 h. Adapted with permission from Ref. [95] Copyright © 2017 Pharmacological Reviews.

Circadian timing in the liver determines the pharmacokinetics of xenobiotics such as drugs. Absorption, distribution, metabolism, and elimination (ADME) in the liver are highly dependent on the part of the day, especially having in mind that cytochrome P450s are rhythmically expressed.[96–98] As a consequence, side effects of drugs can vary significantly if taken during the morning or evening.[50] For instance, even before the chronotherapy field was established, it was observed that administration of chemotherapeutic drugs, such as doxorubicin and cisplatin, in the morning or evening has a significant difference in adverse side effects and therapeutic outcomes.[99] Today, dosing of many chemotherapeutics and the use of radiotherapy are time-restricted.[100–102] The 'clocking the drug' method is also applied in treatments of numerous metabolic diseases such as glucose level control[103] as well as blood pressure regulation.[104]

1.1.1.3 Drugging the clock

When metabolic or signaling pathways are disrupted on the genetic level, time-restricted feeding or following natural rhythms cannot restore their proper functioning. However,

homeostasis may be restored by pharmacologically targeting the circadian clock regulatory components and thereby prevent chronic disease development. Additionally, utilizing small molecules offers one more advantage – a possibility to precisely dose the drug.

As seen from the molecular regulation of the transcription/translation loop, next to the proteins involved in the negative core-clock loop, a wide range of proteins such as kinases are involved in post-translational control.[16,63] This enables two approaches for pharmacological modulating the circadian clocks (Figure 7). The first is direct interaction with the core-clock loop, targeting RORs, CRYs or REV-ERBs proteins.[69,105,106] Secondly, manipulation of the circadian clock can be achieved by modulating post-translational processes. So far, mostly kinases such as CKIα, CKIδ, CKIε, CK2, and GSK-3δ were targeted.[107–109] Interestingly, small-molecule modulators of the circadian rhythm exhibit a wide-range of effects on circadian parameters. For instance, the majority of modulators induce the circadian period change - lengthening[71,105,107,110–117] or shortening,[108,110,113,118,119] while some alter the amplitude[106,120–122] or phase.[120,123,124]

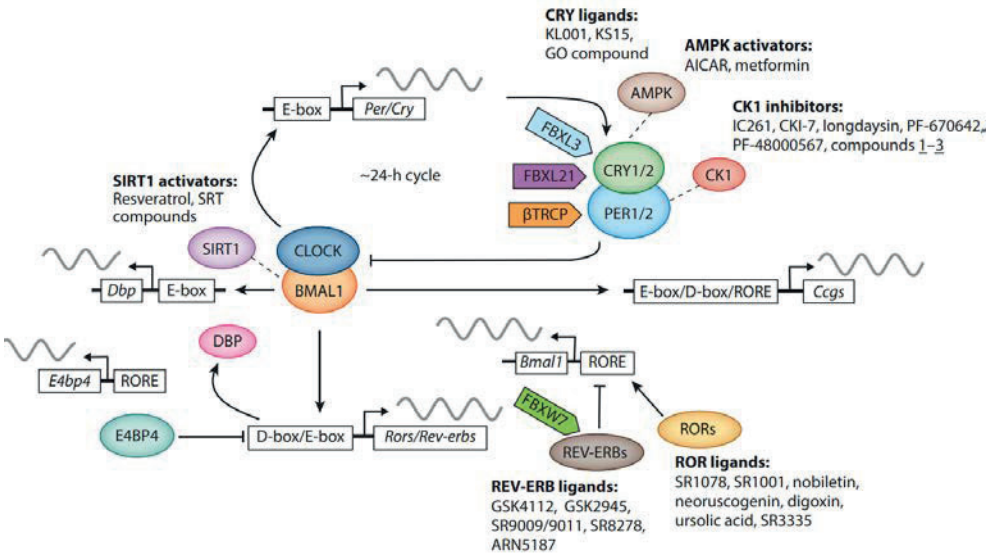


Figure 7. Comprehensive overview of pharmacological ways to modulate the circadian clock. Adapted with permission from Ref. [125] Copyright © 2017 Annual Reviews. All rights reserved.

Considering the research described in this thesis, the main focus in the introduction will be on the period modulators targeting CKI kinase family and CRY proteins.

1.1.6 Small molecule modulators of the circadian period

The development of cell-based high-throughput screening assay by the Kay group in 2008 enabled a fast and approachable discovery of new small molecules that modulate circadian rhythm.[108] The assay is based on U2OS cells harboring reporter genes such as *Bmal-dLuc* and *Per2-dLuc*. In the presence of luciferin, a rhythmically produced luciferase generates bioluminescent output allowing for quantification of circadian expression. A screening of

1280 compounds from the chemical library - LOPAC (Library of Pharmacologically Active Compounds) revealed 13 hit compounds that caused a strong short period phenotype or period lengthening.[108] Most compounds were categorized as inhibitors of protein kinases. A subsequent screening by Ueda and Takahashi groups in 2009, yielded 10 potent period lengthening modulators.[126] Detailed analysis showed that these compounds are mainly inhibitors of CKI (δ and ε) and exhibit a very strong period change in both tested cell lines, central clock tissue and peripheral clock cells. Additional chemical screenings showed that the most significant period-lengthening modulators are predominantly CKI inhibitors (Figure 8).[107,111,112,114,120,127,128] This discovery confirmed the relationship between CKI-dependent phosphorylation of PER and circadian rhythm.

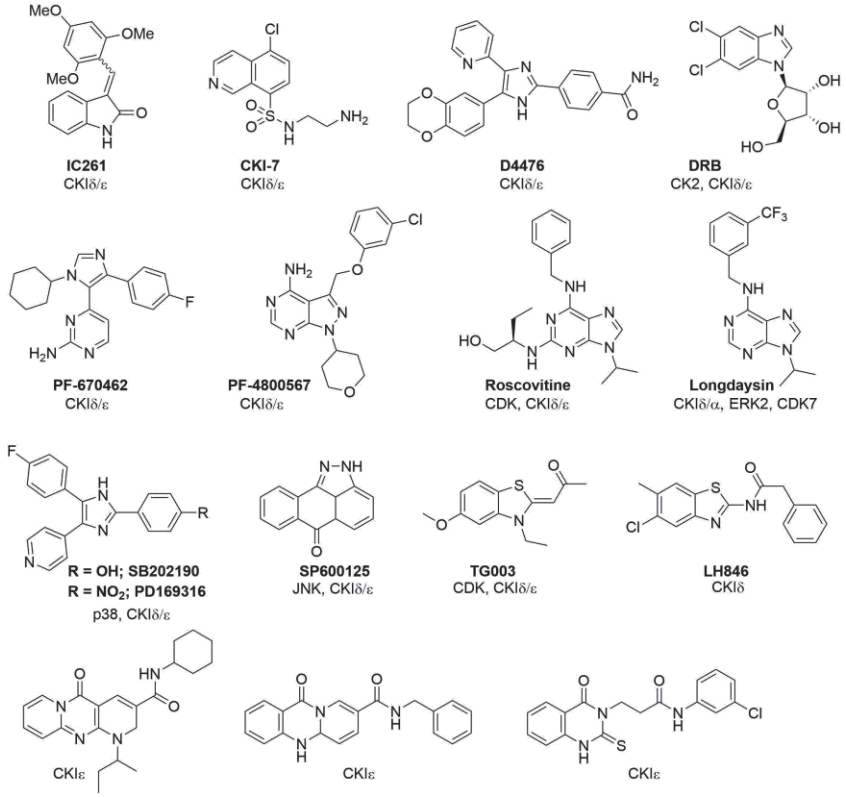


Figure 8. Selected examples of CKI inhibitors with the circadian period lengthening effect.

The first large-scale high-throughput screening (120 000 uncharacterized compounds) performed by the Kay group in 2010 identified a number of modulators with different scaffolds. The purine-based period-lengthening modulator **1** was chosen for further studies because it strongly lengthened the period in dose-dependent manner and had a small effect on the amplitude of oscillations (Figure 9). A brief structure-activity relationship (SAR) study was performed yielding 3-fold more potent compound with more than 10 h period lengthening at a concentration of 10 μM. Due to its pronounced effect it was termed 'longdaysin'. However, longdaysin exhibited a strong period lengthening in both peripheral

and SCN explants, clearly showing that achieving selectivity with compounds modulating the core-clock loop imposes a significant problem for this field. Next, longdaysin was chemically modified into an affinity probe (Figure 9) for the pull-down affinity method coupled with mass spectrometry. Analysis revealed three protein kinases CKIα, CKIδ, and ERK2 (IC_{50} = 5.6, 8.8, 52 μM) as targets. Interestingly, knockdown of each protein kinase had a small period lengthening effect while combined knockdown exhibited the effect similar to the one of longdaysin. This experiment additionally shows the crucial role of small molecules in elucidation of the circadian regulation. In 2019, the same group performed an extensive SAR aiming for a more soluble and potent compound in order to enable mouse behavioral study.[127] Modifying N9 and C2 positions yielded compound NCC007 which is 10-fold more potent than longdaysin and exhibited a period lengthening effect on mouse models (Figure 9).

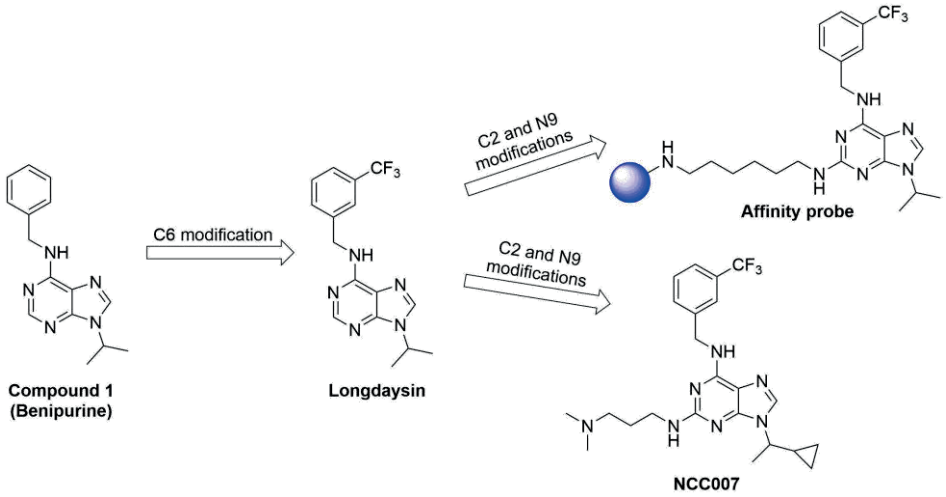


Figure 9. Initially discovered circadian period modulator – benipurine, its more potent analogues longdaysin and NCC007, and the affinity probe.

In 2011, the Shultz and Kay group performed a high-throughput screening of 500 000 compounds.[111] Compound LH846 (Figure 8) was the most potent one with almost no influence on the circadian amplitude. The SAR of 26 compounds was prepared among which compound LH14 was equally potent and LH13 gave the same period change effect at three times lower concentration (Figure 10). Following SAR outcome, the affinity probe was synthesized and LH846 was identified as CKIδ selective inhibitor. These results revealed that CKIδ is more important target for period lengthening effect than CKIε.

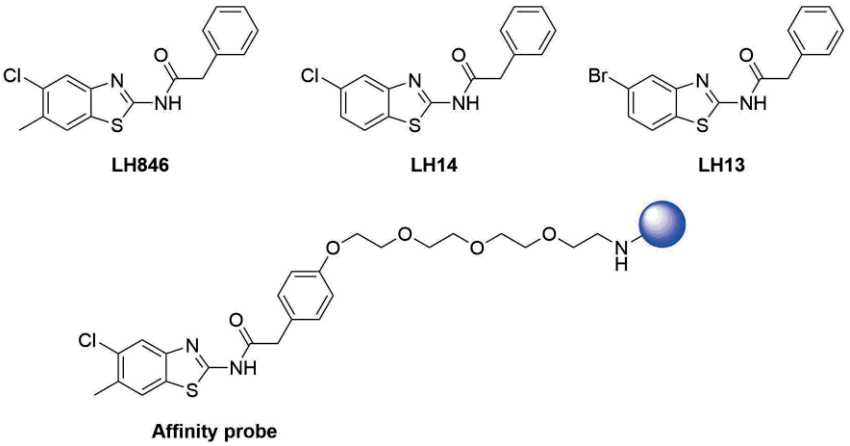


Figure 10. Structures of LH846, LH14, LH13 and affinity probe.

All previously mentioned small molecules targeted protein kinases that post-translationally modify components of the circadian core-clock loop. However, since the constituents of the main negative feedback loop were known, it was thought that targeting some of those proteins would impose better control over the circadian rhythm.

The first small molecule that targets the core-clock loop was a carbazole derivative KL001 discovered from the screening of 60 000 compounds in 2012 (Figure 11).[105] An affinity-based proteomic approach revealed CRY proteins as targets. KL001 exhibited a strong period lengthening in peripheral clocks while the effect in the SCN explant was somewhat reduced but still significant. A year later, the co-crystal structure of KL001 and CRY2 explained that the period lengthening effect comes from competing of KL001 with FAD in the FAD binding site of CRY2. Binding of KL001 to CRY2 causes better stabilization of the protein than FAD and consequently, prevents FBXL3-mediated proteasomal degradation of CRY.[129]

KL001 served as an initial scaffold for the SAR analysis yielding highly active KL044.[130] In another SAR study, the Itami group performed a C–H activation to create a small library of compounds.[110] Remarkably, a relatively small structural change led to period shortening (GO044, GO200, etc.) or period lengthening modulators (GO061, GO214, etc.), implicating on still unknown regulatory mechanism. Recent studies from Kay-Itami-Hirota groups led to discovery of CRY1 selective inhibitor TH129 (the unpublished data).

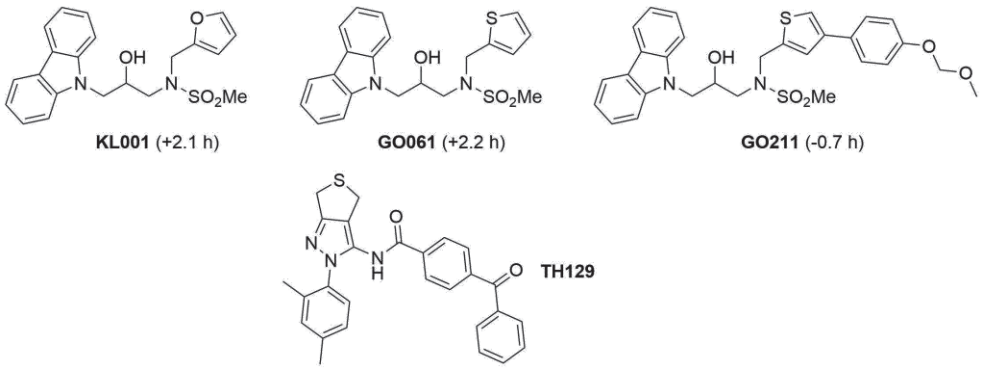


Figure 11. Structures of KL001, GO061, GO211, and TH129. Period effect in hours at a concentration of 10 µM is given in brackets.

1.1.7 Future perspectives and challenges of treating circadian disorders with small molecules

Disruption of the circadian clocks is associated to a growing number of disorders and diseases. Obeying day-night cycles and having time-restricted food intake can synchronize our innate biological time with rotation of the Earth but cannot repair dysfunctional rhythms. Therefore, small molecules offer an irreplaceable approach of modulating and restoring healthy rhythms.

However, despite the fact that a number of small-molecule modulators are already know, there are still major challenges for the field to be overcome, such as:

1. Elucidation of the key interactions between small molecules and proteins involved in the circadian regulation to design more potent drugs
2. Discovery of novel modulators binding to core clock proteins (CRYs, PERs, REV-ERBs, RORs) and therefore targeting the core-clock loop instead of proteins involved in post-translational modifications (CKIs, FBXL3, etc.)
3. Development of suitable, short, cellular, *ex vivo* and *in vivo* assays for utilization of small molecules in elucidation of the circadian regulation as well as connection between clock disruption and disease development
4. Optimization of the target specificity in order to dissect metabolic pathways with higher degree of selectivity and fewer off-target effects
5. Obtaining the spatio-temporal control over the effect of modulators to achieve localized treatment of peripheral or master clock.

Photopharmacology as an emerging field of chemical biology may offer a solution to some of these challenges and contribute to a growing knowledge of circadian regulation and disease development.

1.2 Photopharmacology

Photopharmacology has emerged as the field on the interface of synthesis, photochemistry, and medicinal chemistry.[131,132] It addresses issues caused by the lack of spatial and temporal control over the drug activity. The principle of photopharmacology is based on the incorporation of light-responsive groups into already existing drugs, providing them with the ability to react to light on a structural level. Light is unmatched as an external stimulus due to its non-invasive nature and orthogonality to the most of biological processes.[133] In addition, given that light can be delivered with great accuracy in the human body,[134–136] the effect of photopharmacological agent could be modulated with high spatio-temporal precision by means of activation or deactivation (Figure 12).

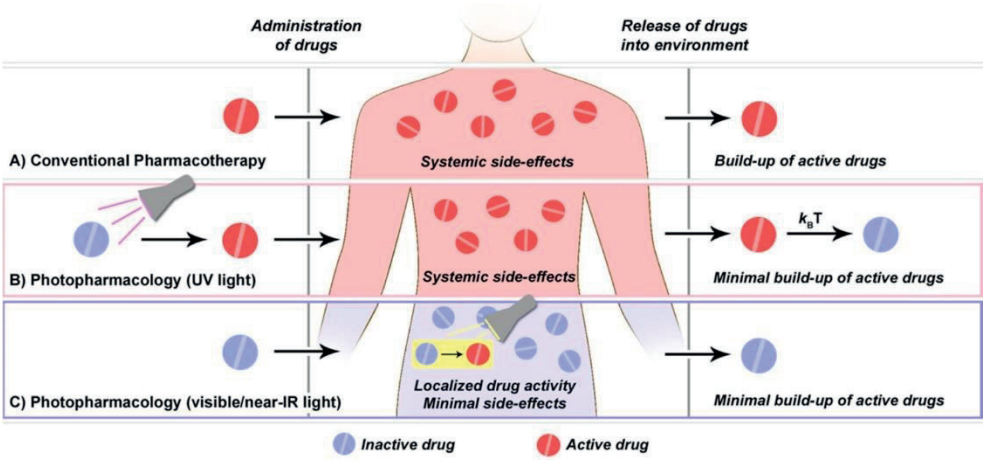


Figure 12. The principle of reversible photopharmacology. Adapted with permission from Ref. [137] Copyright © 2017 American Chemical Society.

The two main strategies to render small molecules photo-responsive utilize photo-removable protecting groups (PPGs or 'cages') and photoswitchable moieties. In the first approach, light induces irreversible structural changes, while the second approach allows for a reversible modulation. During the past decade many biological processes have been successfully addressed using photopharmacology as a tool. For instance, reversible light-induced regulation of bacterial communication and antibiotic resistance buildup,[138,139] ion channels,[140] G protein-coupled receptors,[141] kinases,[142] lipid membranes,[143] and nucleic acids[144,145] has been achieved.

In the context of the main target for the circadian clock modulating in this thesis - CKI kinase family, a short overview on photo-control of the kinase activity will be given.

1.2.1 Protein kinases

Kinases are enzymes that catalyze the transfer of phosphate groups from high-energy phosphate-donating molecules to the substrate.[146] For example, transfer of the γ-phosphate group of ATP is the most common and important process in cell signaling and

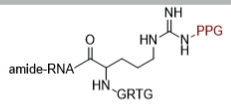
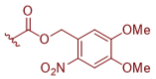
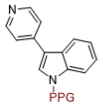
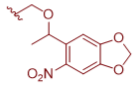
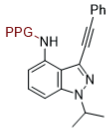
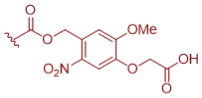
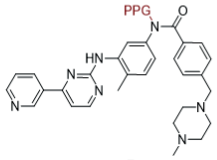
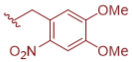
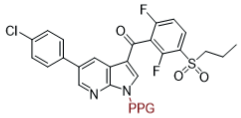
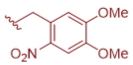
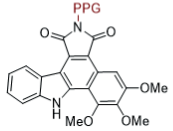
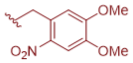
numerous regulatory processes including circadian rhythm. Mutations, overexpression, and dysregulation of protein kinases play a role in the development of numerous diseases, including asthma, inflammatory, autoimmune and nervous system diseases, and, most importantly, cancer.[147] In the last two decades kinases, have been recognized as one of the most important drug targets. Since FDA approved the first kinase inhibitor (imatinib), 47 additional ones were developed and reached the market.[147] Furthermore, more than 5000 crystal structures of kinases have been solved, biological assays are developed for more than 80% of the human kinome, and for more than 20% of kinases small molecule inhibitors were developed.[148]

Kinases can be classified depending on the specificity towards the amino acid substrate, being serine/threonine (Ser/Thr), tyrosine (Tyr) kinases, or having a dual selectivity. The CKI protein kinase family belongs to the Ser/Thr type, and it was the main target for the photo-responsive regulation of the circadian rhythm utilized in this thesis. CKI isoforms are associated with a diverse cellular functions, including DNA replication,[149,150] DNA repair,[151] cytoplasmic-nuclear shuttling of transcription factors,[152] Wnt signaling[153,154] and circadian rhythm.[155,156]

1.2.2 Photocaged kinase inhibitors

PPGs are small molecular entities covalently attached to the drug (in this case kinase inhibitor) and are photo-responsive.[157] Activity of the drug is usually substantially suppressed upon caging because of the significant change in the structure. Light induces removal (uncaging) of the PPG, releasing the inhibitor and restoring the activity. This process is irreversible, and therefore, once uncaged in the target biological system, the small molecule cannot be caged again. Additionally, upon uncaging the released drug will diffuse from the place of irradiation causing decrease in spatial resolution. Therefore, irreversibility imposes one of the main challenges for the utilization of the caged drugs *in vivo*. Yet, numerous caged drugs found the application in biochemistry, neurology and biomedicine for the elucidation of metabolic pathways and regulations.[158–161] An increasing interest in using PPGs led to the discovery of a wide variety photo-responsive groups that can be removed using light of red-shifted wavelengths to prevent application of commonly used, biologically non-innocent UV light.[162] High-energy UV light is known to have a low tissue penetration due to endogenous chromophores absorption[163] and to cause a photodamage to cells.[164] Also, most commonly PPGs are synthetically easy to access and introduce into drugs, causing large structural change and consequently suppressing the activity that can be restored upon light exposure. However, despite the utility of this approach, only a handful protein kinase inhibitors were caged with PPGs (Table 1).[165–169] Interestingly, the first kinase inhibitor was reported in 1998[170] but it took 14 years to design and utilize the next one.[166] PPGs were consistently attached to the nitrogen involved in binding to the corresponding protein kinase, rendering it significantly less active. The largest obtained difference (>200 fold between the potency before and after irradiation) was achieved by protecting VEGFR-2 inhibitor (compound **6**, Table 1). Interestingly, despite having PPGs with extended conjugation and possibility to use visible light (≥ 400 nm),[157] the photo-deprotection was conducted only by UV light. Upon light exposure, release of the active protein kinase inhibitor was accomplished in cell lines but also in zebrafish embryos.

Table 1. A summary of the photocaged kinase inhibitors.

Compound	Structure	PPG	Kinase	Wavelength	Activity (dark)	Activity (light)	Cell line/In vivo model
1			PKA	365 nm	20 μM[a]	420 nM[a]	Rat embryo fibroblast
2			Rho	365 nm	/	/	Zebrafish embryos
3			RET	365 nm	6.8 μM[b]	590 nM[b]	Zebrafish embryos
4			PDGF-R	365 nm	5.8 μM[b]	8.9 nM[b]	/
5			BRAF	365 nm	440 nM[c]	10.2 nM[c]	SkMel13 melanoma cell line
6			VEGFR-2	365 nm	34.6 μM[b]	0.158 μM[b]	VEGFR-2 dependent PC-3 cells

[a] K_i; [b] IC_{50}; [c] K_d

One of the goals in this thesis was the development of a caged kinase inhibitor that can be used for a precise control of the kinase activity involved in post-translational modifications of the circadian core-clock loop. Ideally such inhibitor should be responsive to visible or near infrared light.

1.2.3 Photoswitchable kinase inhibitors

Spatio-temporal control in the approach that utilizes PPGs is limited due to its irreversibility and diffusion. Once uncaged, the active drug will diffuse, decreasing the spatio-temporal resolution. Thus, incorporation of a reversible photochromic moiety into kinase inhibitors can potentially allow for a higher resolution and better understanding of the complex cellular regulation.[131,132,171] The most commonly applied molecular photoswitches in photopharmacology are azobenzenes and diarylethenes (Figure 13).

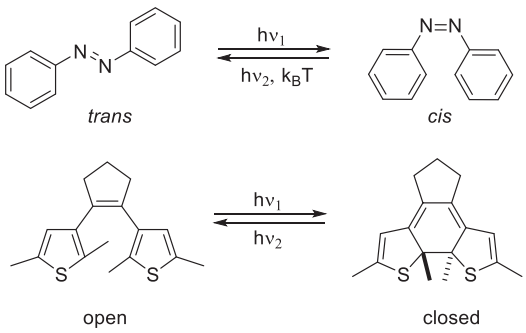


Figure 13. Photoisomerization of azobenzenes and diarylethenes.

Azobenzenes are one of the best studied chromophores and therefore, most commonly a photoswitch of choice. Configurational change between the two isomers – *trans* (stable) and *cis* (less stable) can be induced by light in both directions, while the *cis*-to-*trans* process is also thermally driven, making this system reversible. Furthermore, the geometrical change upon switching is accompanied by a large change in the dipole moment, ~3 D.
Diarylethenes undergo a light-induced reversible cyclization, causing a change in rigidity and electronic properties of the molecule. Generally, both forms (open and close) are thermally stable and can be interconverted by light of different wavelength.[131]

Similarly to the low number of caged kinase inhibitors, only six inhibitors have been rendered photo-switchable. In 2015, the first example of a photoswitchable small molecule kinase inhibitor was published by the Grøtli and Andréasson groups (Figure 14).[142] The original inhibitor **7** of rearranged during transfection (RET) kinase, a type of transmembrane receptor tyrosine kinase, was rendered photoswitchable by exchanging the alkyne for an azo moiety. The *trans*-to-*cis* isomerization was conducted by UV light (λ_{max} = 365 nm) and back isomerization was achieved using green light (λ_{max} = 503 nm) or thermally. *In vitro* assay showed 3.8-fold stronger inhibition of the *trans*-**8** while the effect in the cellular assay was slightly lower *i.e*. 3.2 fold.

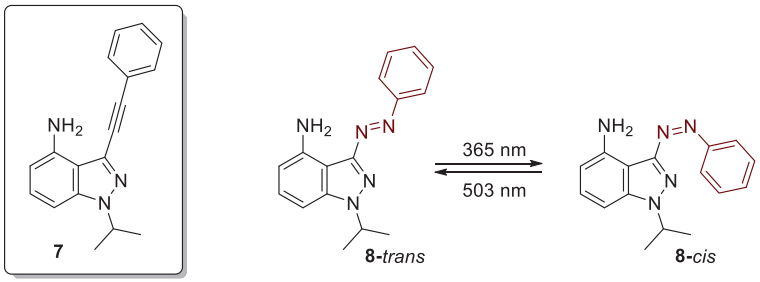


Figure 14. Initial structure of RET kinase inhibitor **7** and photoswitching of its azolog **8**.

A subsequent example of a photoswitchable kinase inhibitor comes from the Branda group.[172] Compound **9**, a known inhibitor of protein kinase C (PKC), served as a starting point for development of the photo-responsive analogue **10** (Figure 15). The bisindolylmaleimide (BIM) structure of **9** contains the 1,3,5-hexatriene backbone, but, due to a distortion from s-*cis*-s-*cis* conformation required for a photochemical ring-closing, this molecule could not be used as a photoswitch. Thus, one indole heterocycles was replaced by a thiophene ring to enhance the photocyclization reaction. The s-*trans*-s-*trans* (**10**-*i*) conformer mimics the known inhibitor serving as the active form, but it features an undesired arrangement of the hexatriene backbone for the photocyclization. In order to photoisomerise to the inactive closed form (**10**-c), a rotation to form the s-*cis*-s-*cis* (**10**-o) conformer is required. However, the photoinduced ring-closing is highly suppressed in polar solvent such as water due to the presence of a twisted intramolecular charge transfer state populated upon photo-excitation. Therefore, the authors had to perform the photocyclization reaction in THF, reaching only 42% of the closed form (**10**-c). Conveniently, isolated **10**-c form underwent photo-induced ring-opening in *in vitro* medium, leading to the activation. The active **10**-i form showed an IC_{50} of 580 nM. However, the main drawback of this approach was that is was not possible to perform reversible light-control over PKC activity in aqueous medium.

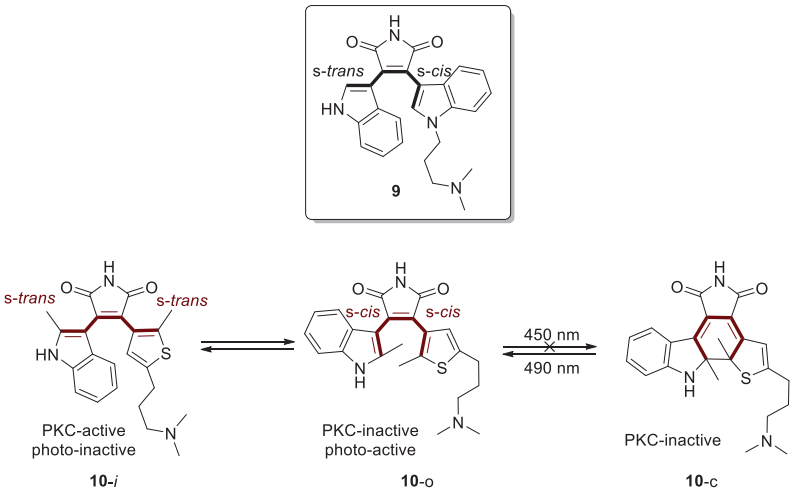


Figure 15. A known PKC inhibitor **9**, and the photoisomerization of its analogue **10**.

By employing genetically-directed bioorthogonal ligand tethering (BOLT) approach, the Chin group enabled both isoform-selective and optically controlled mitogen-activated kinase (MEK).[173] MEK1 and MEK2 are key kinases of cell division, growth and development, being a part of the MAP kinase signaling pathway. Dysregulation of this pathway is observed in numerous types of human tumors, but selective MEK1/2 inhibition has never been achieved.

The azetidine moiety of MEK1/2 inhibitor **11** was replaced by a photoswitchable linker that was further covalently attached to a modified amino acid residue **12** on the protein surface of MEK1 (Figure 16). This allowed for a reversible control of the MEK1 activity by light. In its thermodynamically stable *trans*-form, the inhibitor can reach the binding site, inhibiting MEK1. Photo-isomerization of the linker by UV-light (λ_{max} = 365 nm) changes the shape of the whole molecular construct, preventing the inhibitor from binding and consequently restoring catalytic activity.

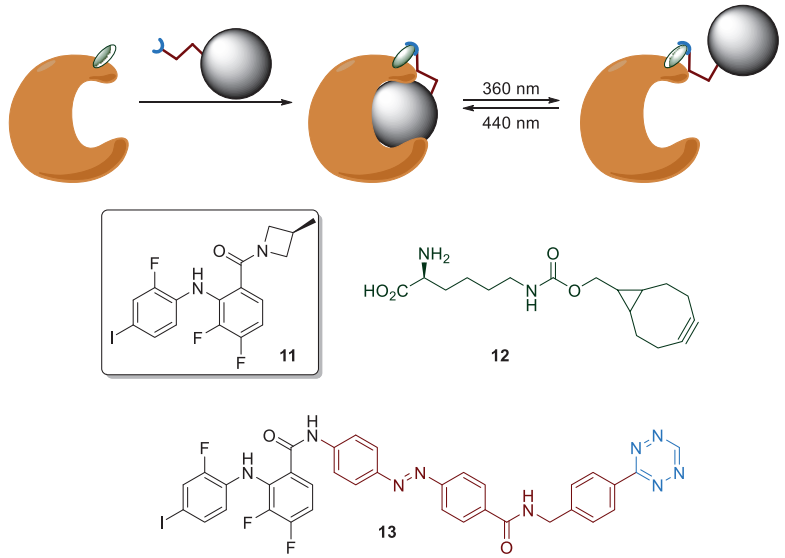


Figure 16. The principle and design of the BOLT approach and components of the photoswitchable molecular construct.

Schmidt *et al* have identified a photo-responsive stilbene moiety of axitinib as a potential photoswitch for a reversible modulation of its VEGFR2 (vascular endothelial growth factor receptor 2) inhibition (Figure 17).[174] To test a hypothesis that the stilbene moiety can be used as a photoswitch, photoisomerization studies were performed in DMSO and water. Axitinib underwent reversible photoisomerization in DMSO, while in water the isomerization was unidirectional, with *Z*-to-*E* switching being almost quantitative (>90% of *E*). *E*-to-*Z* switching in aqueous medium was not successful due to the competing irreversible [2+2]-photocycloaddition reaction leading to the inactive dimer **14**. However, the advantage of this photochromic system is that both isomers of axitinib are thermally stable and separable by column chromatography. After separation, both isomers were tested in the VEGFR2 kinase assay. Interestingly, the *Z*-isomer was 43 times less active and

the biological activity of *E*-axitinib could be almost completely restored upon exposure of *Z*-axitinib to UV light (λ_{max} = 385 nm). On the other hand, upon exposure of *Z*-axitinib to the same wavelength in the cellular HUVEC proliferation assay, it was found that the inhibitory activity increased only by a factor of 2.6, suggesting that the *Z*-to-*E* photoisomerization was not efficient. Despite showing a promising difference in activity between the isomers, due to an irreversible dimerization in aqueous medium, *E*-to-*Z* switching was not feasible and therefore not performed in a biological context. These results show that axitinib can be employed as a photoswitch but unfortunately, only one-way and with limited efficacy *in cellulo*.

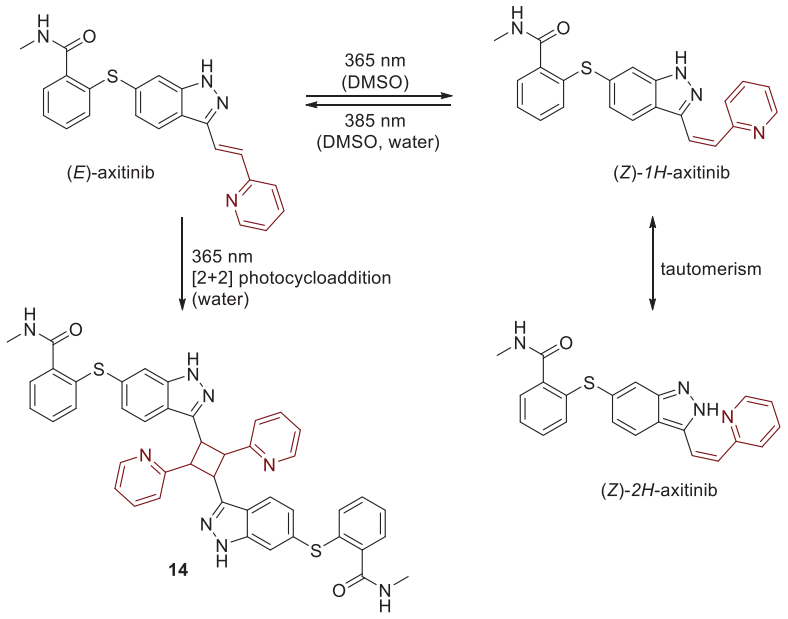


Figure 17. Photochemically driven processes of axitinib in DMSO and water.

The structure of highly potent p38α MAPK and CKIδ inhibitor **15** was also subjected to modification with photoswitchable moieties such as arylazo (**16** and **17**) and diazocine (**18**) (Figure 18).[175] Considering a fast thermal *Z*-to-*E* isomerization of arylazoimidazoles,[176] the imidazole moiety of the original inhibitor **15** was methylated (**16**) or replaced with thiazole (**17**) in order to extend the half-life of the less stable *Z*-isomer. Furthermore, diazocines exhibit higher stability in the *Z*-configuration[177] and prolonged thermal half-life of a thermodynamically less stable *E*-isomer. Surprisingly, compound **17** did not inhibit p38α or CKIδ, while photoswitch-modified inhibitors **15** and **16** exhibited high potencies within the nanomolar range but with no light-induced effect. After a detailed analysis and solving the crystal structure of ligand **15** with p38α, it was discovered that both heterocyclic azobenzenes underwent reduction to the corresponding hydrazines. The azo group was found to be reduced by dithiothreitol (DTT), a protein-stabilizing agent used in biological assays. The absence of photo-responsive azo group explained why inhibitors **15** and **16** did not exhibit light-dependent effect.

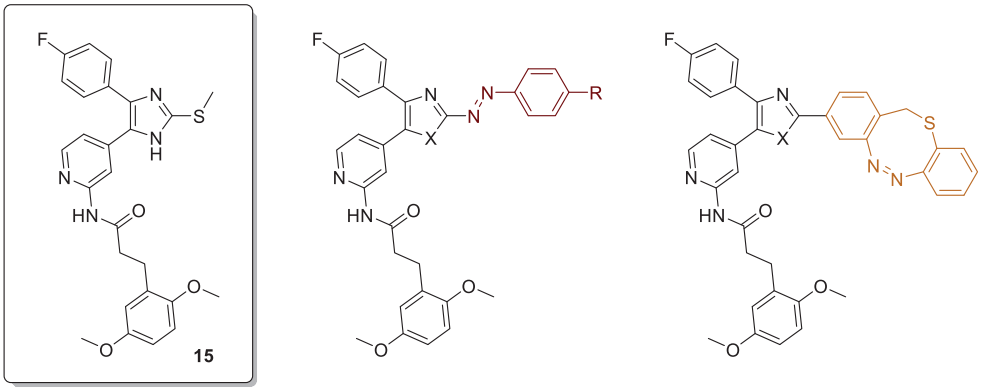


Figure 18. A known CKIδ/p38α MAPK inhibitor **15** and its photoswitchable analogues **16**, **17** and **18**.

The most recent example of successfully designed and applied photoswitchable kinase inhibitor was presented by Hoorens *et al*.[178] In this study, the sulfonamide moiety of $BRAF^{V600E}$ inhibitor **19** was identified as a potential group for azologization. A small library of 8 photoswitches was prepared and tested in an assay with purified recombinant $BRAF^{V600E}$ with inactive MEK-1 as a substrate. Despite structural similarity throughout the SAR library, only photoswitch **20** displayed a significant increase in activity upon *trans*-to-*cis* photoisomerization (10-fold). Interestingly, upon UV light exposure, thermodynamically stable *trans*-**21** was almost quantitatively converted to the *cis*-isomer in comparison to only 55% in the case of **20**. However, photoswitch **21** exhibited almost the same activity in both conformations, emphasizing the importance of controlling almost all chemical and photochemical parameters in order to achieve a successful photopharmacological control over kinases. The lack of photo-modulation with photoswitch **21** can be possibly ascribed to a narrow binding site of $BRAF^{V600E}$ which could not accommodate an additional methoxy group, or to a short thermal half-life of *cis*-**21** in the kinase assay medium. The authors determined a reasonable thermal half-life of **21** only in 1:1 mixture of acetonitrile and kinase assay medium (>24 h), while it could be much shorter in a pure medium, especially keeping in mind the push-pull character of the azobenzene.

In order to rationalize a choice for azologization and biological results, docking studies were performed. Docking showed high structural similarity between the conformer *cis*-**20** and the sulfonamide, explaining why photoisomerization of the more thermodynamically stable *trans*-isomer to the *cis*-isomer led to activation.

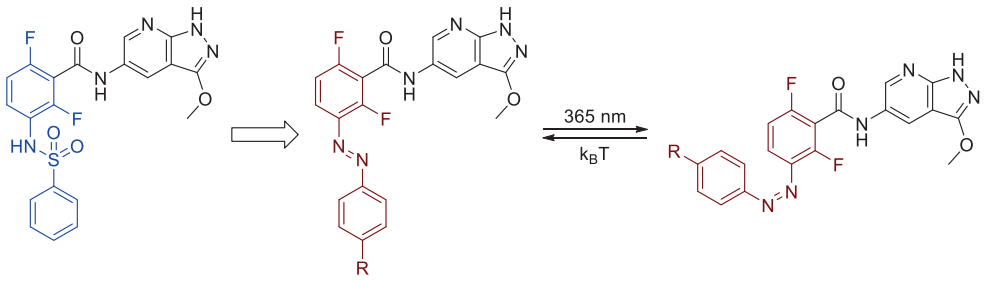


Figure 19. Sulfonamide-based $BRAF^{V600E}$ inhibitor **19**, its azologs **20** and **21**, and *trans*-to-*cis* photoisomerization with UV light.

The presented and very limited set of known photoswitchable kinase inhibitors clearly illustrates multiple difficulties (cycloaddition of stilbenes, reduction of the azo group, photochemically irreversible processes in aqueous medium, etc.) in creating fully functional small molecule that can reversibly modulate activity of kinases with light.

Additionally, it is interesting to notice that, similarly to the caged kinase inhibitors, there are no photoswitchable inhibitors that employ longer wavelengths (e.g. green or red light) for the isomerization and yet are able to efficiently modulate the kinase activity.

1.3 Challenges for chronophotopharmacology

The use of photoswitches to obtain dynamic and reversible control over biological processes, and especially over the circadian rhythm, requires numerous parameters to be taken into account and optimized at the molecular level. The length of circadian assays (5 or more days),[107,120] together with the property of the *cis*-isomers to thermally isomerize back to the *trans*-isomer, renders the utilization of purely light-induced manipulation of the circadian period particularly challenging. The circadian assay requires photochemical and chemical properties to be optimized in order to achieve a noticeable effect and suppress the background activity of each isomer. While in other photopharmacological applications certain photochemical properties might be less important (photostationary state, thermal half-life, etc.),[179–181] here almost all parameters had to be taken into consideration. General drug features that had to be retained as well as photochemical parameters that have to be optimized for reversible modulation of the circadian period (and biological activity in general) are the following:

1. **Retaining potency, solubility, and selectivity upon incorporation of the photoswitch**. Most commonly, rendering small molecules photoswitchable by incorporation of the azobenzene moiety results in decreased solubility and potency. Drugs are exceedingly optimized structures and incorporation of new moieties usually lowers the potency.[131] This can impose a difficulty in reaching the effective pharmacological concentration, especially in the case when decrease in solubility is accompanied by decrease in potency, and when application in *ex vivo* or *in vivo* system is envisioned. The drugs are also highly optimized toward selectivity and minimizing off-target effect. Therefore, retaining target-selectivity imposes an additional challenge. This issue is particularly pronounced when the target is a kinase due to greatly conserved catalytically active domain (where the ATP-binding pocket is located) throughout the kinome.[148,182,183] Thus, taking highly selective kinase inhibitor to render it photoswitchable is essential in order to utilize this system for understanding of the target biology and prevent off-target effect. In this thesis, longdaysin[107] and LH-derivatives[111] were the kinase inhibitors of choice due to their pronounced selectivity towards casein kinase I family - one of the main post-translational regulators of the circadian (Figure 5);
2. **Retaining tunable (switchable) activity** in biologically relevant media, cells, tissues and living organisms.
3. **Maximizing the difference in binding affinity between the two isomers**. In order to achieve a distinct biological difference between the two photo-isomers, the difference in activity is preferred to be as large as possible;
4. **Adjusting the thermal half-life of the less thermodynamically stable isomer**. In most cases the *cis*-isomer is less stable and undergoes spontaneous thermal back-isomerization to the *trans*-isomer. In longer biological assays, in particular in the circadian, even with the highest amount of the *cis*-isomer in the PSS, fast thermal back-isomerization will prevent a distinguished effect of both isomers to be observed;
5. **Achieving the highest possible photostationary state (PSS)**. PSS is one of the key parameters in design of photoswitchable drugs. It indicates a practical efficiency of the

photoswitch upon irradiation at certain wavelength, and is usually presented as a ratio or percentage of one isomer in respect to the other. Unfortunately, for most of photoswitches the photoisomerization does not lead to a light-induced quantitative interconversion between isomers. Since commonly both isomers are biologically active, albeit with different level of potency, the PSS has to be as high as possible in order to minimize a background activity of the undesired isomer. Having high PSS distributions becomes a crucial parameter especially when the *trans*-form is the more active isomer and a biological effect is suppressed by irradiation. The circadian assays commonly take 5-6 days,[108] thus when the effect of the *cis*-isomer is measured, it is crucial to start with the highest possible PSS distribution and retain it for as long as possible. If this is not the case, the thermal relaxation during such a long period of time will diminish the effect of the *cis*-isomer and prevent quantifying it.

6. **(Photo)chemical stability in biological assays**. Avoiding oxidation or reduction of the photochromic functional group in biological medium is of crucial importance, especially if the effect is measured during longer periods like in the circadian asays.[175] The isomerization is mediated by light as an external energy input. However, the energy input does not necessary need to lead only to a desired photoisomerization reaction, but the energy-reach intermediate can react in various other ways such as [2+2]-cycloaddition in case of stilbenes[174] or when intersystem crossing competes with photoswitching.[184] The later process can lead to the formation of singlet oxygen which is toxic to the biological surrounding or causes bleaching of the chromophore.[144,185,186] Luckily, photoisomerization of the most commonly applied azobenzenes is on the time scale of picoseconds, preventing the formation of singlet oxygen.[132]
7. **Enabling a visible light photoisomerization in both directions**. The vast majority of photoswitches biologically applied today utilize UV light for *trans*-to-*cis* isomerization. While it is feasible to use UV-light-induced isomerization for biological regulation, for application in *ex vivo* and *in vivo* studies this is a limiting factor. UV light is cytotoxic, has a negligible tissue penetration and is poorly biocompatible, while visible and infrared light are biorthogonal and have larger tissue penetration.[187] In addition, the circadian bioluminescent assays usually rely on luciferin-reach medium,[108] and it strongly absorbs UV light preventing photoisomerization during the assay;
8. **Efficiency of the photochemical isomerization**. In order to increase efficiency the molar absorptivity and the quantum yield of the isomerization process has to be the highest possible.

Considering all these parameters and challenges, a reversible, (photo)chemically stable and visible light responsive kinase inhibitor would impose a state-of-the-art molecular entity for controlling and understanding of biological processes through the circadian clock regulation.

1.4 Aim and outline of this thesis

A major challenge in the pharmacological approach of chronotherapy is to control the circadian period locally. The difficulty emerges from the uniform cellular regulation of the circadian rhythmicity throughout the whole mammalian body. Hence, this thesis describes the development of the first photo-responsive small molecule modulators of the biological clock with the aim in obtaining high spatio-temporal control over the circadian period lengthening.

Chapter 1 introduces the circadian rhythm, diseases and disorders linked to its disruption and methods in restoring healthy rhythms. The emphasis is on discovery and development of small molecule modulators for the pharmacological modulation of the circadian rhythm. In addition, this chapter introduces photopharmacology as a tool for rendering drugs photo-responsive. By merging fields of chronotherapy and photopharmacology, we aspired to create a new filed of chronophotopharmacology in which activity of the circadian period modulators can be controlled by light. The major challenges of chronophotopharmacology are depicted at the end of this chapter.

Chapter 2 describes the first synthetic approach to obtain 6-azopurines. Heterocyclic azobenzenes were obtained in microwave-assisted nucleophilic aromatic substitution followed by metal-free oxidation with oxygen. Next to a straightforward two-step-one-pot synthesis, photochemical studies were conducted and revealed a strong n-π* transition which allowed to use green light (λ_{max} = 530 nm) for *trans*-to-*cis* isomerization. Purine-based structure of these molecular photoswitches, good photochemical stability, and the possibility to conduct photoisomerization with green light make them promising candidates for the reversible modulation of biological processes.

Chapter 3 shows the utilization of photoswitches prepared in Chapter 2 for the reversible modulation of the circadian period by light. In view of the biological application, next to the known photochemical properties in DMSO (Chapter 2), photochemistry in biological media (kinase assay buffer and cellular medium) was studied. DTT-containing kinase assay buffer revealed that the azo group undergoes reduction to the corresponding hydrazine. The resulting light non-responsive hydrazines prevented photo-modulation of the circadian rhythm. Yet, the structure-activity relationship was performed. It showed that replacing *meta*-CF_3 group with two *meta*-chloro-substituents, and introducing 2-amino group increase potency.

In Chapter 4, another class of photoswitches based on longdaysin structure was prepared. In order to circumvent the reduction of the photochromic moiety, the design did not involve heterocyclic azobenzenes. A thorough investigation of the structure-activity relationship on the enzymatic and cellular level led to the identification of crucial photochemical parameters for the circadian clock modulation with light. Ultimately, the first visible-light operating circadian period modulator based on tetra-*ortho*-fluoro azobenzene moiety was made.

Chapter 5 presents of a small library of acyl-hydrazone photoswitches based on circadian period modulator LH14. Photochemical properties and biological activity were investigated.

Remarkably, the methyl group had a significant outcome on the biological activity of envisioned modulators.

In Chapter 6, longdaysin was protected with a photoremovable protecting group (PPG) in order to enable light-inducible regulation of the clock function via CKI activity control. After recognizing the crucial interaction between longdaysin and CKI, the activity of this kinase inhibitor was silenced by the incorporation of different PPGs. Light irradiation enabled the inhibitory activity to be restored. The circadian period was successfully modulated with high temporal precision on the cellular and tissue level, as well as in zebrafish.

Chapter 7 shows the attempt to create a new class of photoswitchable circadian modulators that are more potent in their *cis*-form. The benzophenone moiety of the CRY inhibitor TH129 was recognized as a potential structure for azologization. Our rational design is further confirmed by molecular modelling. In contrast to the previous light-responsive modulators, these photoswitches control the circadian period directly interacting with the core clock loop and not post-translational modifications of the clock proteins.

1.5 References

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